

# Analysis of Blood Specimens for Anticoagulant Rodenticides by LC-FTMSMS

## 1 Introduction

A series of compounds structurally related to 4-hydroxycoumarin have been used for many years as rodenticides. All of these compounds work by inhibiting the vitamin K epoxide reductase enzyme, leading to a decrease in circulating levels of vitamin K. This, in turn, causes a drop in blood levels of clotting factor VII and prothrombin, both of which are necessary for formation of blood clots. These compounds also tend to increase the permeability of the walls of blood vessels, leading to uncontrolled internal bleeding upon receipt of toxic doses of any of these compounds. In mid-2011 the U.S. Environmental Protection Agency moved to ban the use of the "second generation" anticoagulant rodenticides, including bromadiolone, brodifacoum, and difenacoum, in consumer products intended for residential application, although they remain in use for products intended for outdoor and commercial use. The "second generation" compounds have a higher toxicity, both acute and chronic, than older compounds such as warfarin, coumachlor, and coumatetralyl. Warfarin is also used, under the trade name Coumadin, as a therapeutic anticoagulant for prevention of thrombosis and embolisms.

## 2 Scope

This procedure qualitatively identifies six anticoagulant rodenticides in whole blood. It is derived from "The Determination of the Anticoagulant Rodenticide Brodifacoum in Blood Serum by Liquid Chromatography with Fluorescence Detection" and "Acute Bromadiolone Intoxication" published in the Journal of Analytical Toxicology in 1989 and 2006, respectively. The extraction procedure has been adapted from the *Analysis of Blood and Urine Specimens for THC and 11 COOH THC* standard operating procedure (Tox 405). Protein precipitation with acetonitrile is coupled with a solid phase cleanup and subsequent analysis of the isolate by LC-FTMSMS.

## 3 Principle

Blood and specimens may be screened and/or confirmed for brodifacoum, bromadiolone, coumachlor, coumatetralyl, difenacoum, and warfarin by this method. Blood samples are protein precipitated with acetonitrile followed by solid-phase extraction with Bond Elute Certify II cartridges. The resulting extract is taken to dryness and analyzed by LC-FTMSMS in the positive electrospray ionization mode.

## 4 Specimens

This procedure uses two 1 mL portions of blood. It can also be adapted for analysis of rat bait pellets and other commercial products. When analyzing commercial samples, a 20-fold dilution or 20x homogenate in deionized water (DI) will typically be used.

## 5 Equipment/Materials/Reagents

Guidance for preparing reagents may be found in the *Preparation of Chemical Reagents* standard operating procedure (Tox 103).

- a. Screw-top test tubes with caps
- b. Culture tubes with caps
- c. Volumetric flasks (10 mL and 100 mL)
- d. Quantitative pipetters covering the volume range 0.02-1.0 mL, with disposable tips
- e. Qualitative pipetter with disposable tips and/or glass serological pipets (0.5-5.0 mL range)
- f. Vortex mixer
- g. Centrifuge
- h. Evaporator with nitrogen
- i. SPE manifold (vacuum or positive pressure)
- j. Bond Elut Certify II solid-phase extraction cartridges
- k. Liquid chromatography-high resolution (30000 FWHM) mass spectrometry system equipped with a 15 cm x 2.1 mm x 5  $\mu$ m d<sub>p</sub> Grace Altima C18 (or equivalent) column
- l. Routine laboratory supplies, including: Pasteur pipets, pH paper, graduated cylinders, etc
- m. Acetonitrile (HPLC grade)
- n. Toluene (HPLC grade)
- o. Methanol (HPLC grade)

- p. Deionized water (DI)
- q. Sodium Acetate Buffer (0.1 M, pH 7)
- r. Sodium Acetate Buffer (0.1 M, pH 7) with 5% Methanol
- s. Rodenticides Wash Solvent (95:5 hexane:ethyl acetate)
- t. Rodenticides Elution Solvent (75:25:1 hexane:ethyl acetate:acetic acid)
- u. Methanol:water (1:1)
- v. Rodenticides LC Mobile Phase #1 (0.06% acetic acid in water). Stable for a maximum of 2 weeks; do not extend expiration date.
- w. Rodenticides LC Mobile Phase #2 (0.06% acetic acid in methanol). Stable for a maximum of 2 weeks; do not extend expiration date.

## 6 Standards and Controls

- a. Brodifacoum:  
Obtained as a solid from an approved vendor. Stability and storage conditions determined by manufacturer.
- b. Bromadiolone:  
Obtained as a solid from an approved vendor. Stability and storage conditions determined by manufacturer.
- c. Coumachlor:  
Obtained as a solid from an approved vendor. Stability and storage conditions determined by manufacturer.
- d. Coumatetralyl:  
Obtained as a solid from an approved vendor. Stability and storage conditions determined by manufacturer.
- e. Difenacoum:  
Obtained as a solid from an approved vendor. Stability and storage conditions determined by manufacturer.
- f. Warfarin Stock Solution (1 mg/mL in methanol):  
Obtained from Cerilliant or another approved vendor. Stability and storage conditions

determined by manufacturer.

- g. Brodifacoum Stock Solution (1 mg/mL):  
Weigh 10 mg of brodifacoum into a 10 mL volumetric flask, and add 5 mL of toluene and 4 mL of methanol. Mix to dissolve and fill to the mark with methanol. Store in glass at <0°C. Stable for at least 1 year.
- h. Bromadiolone Stock Solution (1 mg/mL):  
Weigh 10 mg of bromadiolone into a 10 mL volumetric flask, and add 5 mL of toluene and 4 mL of methanol. Mix to dissolve and fill to the mark with methanol. Store in glass at <0°C. Stable for at least 1 year.
- i. Coumachlor Stock Solution (1 mg/mL):  
Weigh 10 mg of coumachlor into a 10 mL volumetric flask, and add 5 mL of toluene and 4 mL of methanol. Mix to dissolve and fill to the mark with methanol. Store in glass at <0°C. Stable for at least 1 year.
- j. Coumatetralyl Stock Solution (1 mg/mL):  
Weigh 10 mg of coumatetralyl into a 10 mL volumetric flask, and add 5 mL of toluene and 4 mL of methanol. Mix to dissolve and fill to the mark with methanol. Store in glass at <0°C. Stable for at least 1 year.
- k. Difenacoum Stock Solution (1 mg/mL):  
Weigh 10 mg of difenacoum into a 10 mL volumetric flask, and add 5 mL of toluene and 4 mL of methanol. Mix to dissolve and fill to the mark with methanol. Store in glass at <0°C. Stable for at least 1 year.
- l. Rodenticides Working Solution (1 µg/mL of each component)  
Combine 100 µL each of the warfarin, brodifacoum, bromadiolone, coumachlor, coumatetralyl, and difenacoum stock solutions in a 100 mL volumetric flask, fill to the mark with methanol, and mix well. Store refrigerated in glass. Stable for at least 3 months.
- m. Rodenticide LC/MSMS Performance Mix (0.05 µg/mL):  
Mix 50 µL of the rodenticides working solution with 950 µL of the rodenticides LC mobile phase #2. Prepare fresh daily.
- n. Negative Control Blood:  
Blood is purchased from Cliniqa or another approved vendor. Storage and stability determined by manufacturer. A Negative Control Blood sample will be extracted and analyzed with every blood assay.

- o. **Positive Control Blood:**  
Prepared at 25 ng/mL by spiking 1 mL of Negative Control Blood with 25 µL of the Rodenticides Working Solution and at 100 ng/mL by spiking 1 mL of Negative Control Blood with 100 µL of the Rodenticides Working Solution. Positive Control Blood samples will be extracted and analyzed with every blood assay. Additionally, when sample volume permits, a 1 mL portion of the case specimen to be analyzed will be fortified with 25 µL of the rodenticides working solution to demonstrate recovery from that specific matrix.

## 7 Calibration

Not applicable.

## 8 Sampling

Not applicable.

## 9 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the examiner or chemist performing the procedure.

- a. Into properly-labeled test tubes, add 1 mL of control or case samples and enough DI water to bring the volume to 1.2 mL. Prepare in duplicate if specimen volume allows in order to prepare a Positive Control Blood sample as directed in Section 6 above.
- b. Add 4 mL of acetonitrile drop wise while vortexing sample. Vortex thoroughly for a minimum of 3 minutes.
- c. Centrifuge samples for 15 minutes.
- d. Transfer the supernatant to a clean test tube and evaporate sample to about 2 mL under a slow stream of nitrogen at approximately 50°C.
- e. Add 6 mL of 0.1 M sodium acetate buffer (pH 7).
- f. Prepare the Bond Elut Certify II columns by sequentially passing 2 mL methanol and 2 mL 0.1 M sodium acetate buffer (pH 7) with 5% methanol. Do not allow sorbent bed to dry.
- g. Pour the sample into the appropriately labeled column reservoir. Draw the sample through

the column at a flow rate of approximately 1 - 2 mL/minute.

- h. Rinse column with 1 mL of 0.1 M sodium acetate buffer (pH 7).
- i. Dry column under full vacuum for 1 minute.
- j. Sequentially rinse column with 2 mL of Rodenticide Wash Solvent followed by 5 mL of methanol:deionized water (1:1).
- k. Dry column under full vacuum for 1 minute.
- l. Elute rodenticide fraction: Place rack with labeled tubes in the SPE manifold and wipe tips of needles. Slowly elute with 2 mL of the Rodenticide Elution Solvent at about 1 mL/minute. Evaporate under nitrogen at approximately 50°C.
- m. Reconstitute dried extract in 100 µL of Rodenticide Mobile Phase #2.
- n. Analyze 10 µL of the LC/MS Performance Mix to determine that the LC/MS is in proper working condition.
- o. Analyze 10 µL of each extract by LC-FTMSMS.

## 10 Instrumental Conditions

Following are the instrumental parameters used in this procedure:

### 10.1 Liquid Chromatograph Parameters

Mobile Phase Compositions	Flow Parameters			Column Parameters	
1: Water with 0.06% Acetic Acid	flow rate	0.3 mL/min		type	C-18
	time (min)	%1	%2	length	15 cm
2: Methanol with 0.06% Acetic Acid	0.0	22	78	internal diameter	2.1 mm
	3.0	22	78	particle size	5 µm
	8.0	5	95	temperature	40°C
	20	5	95		
	21	22	78		
	28	22	78		

## 10.2 Mass Spectral Parameters

3 Segments		
Segment 1 – 0-5 minutes – 2 scan events		
Event #1	full scan m/z 240-400 profile at 30000 resolution	
Event #2	MS/MS at 7500 resolution	collision energy: 30 (rel) for 343.073 and 309.112; 40 (rel) for 293.117
	precursor from cyclic scan table: 2-5 min for m/z 293.117 and m/z 343.073; 1.3-4.3 min for m/z 309.112	
	isolation width: 3.0 AMU	scan range: software control
Segment 2 – 5-13 minutes – 2 scan events		
Event #1	full scan m/z 390-580 profile at 30000 resolution	
Event #2	MS/MS at 7500 resolution	collision energy: 30 (rel)
	precursor from cyclic scan table: 8-11.5 min for m/z 445.180; 5.5-9 min for m/z 509.075; 9-12.5 min for m/z 523.090	
	isolation width: 3.0 AMU	scan range: software control
Segment 3 – 13-28 minutes – 1 scan event		
Event #1	Full scan m/z 240-580 profile at 30000 resolution	

NOTE: The precursor ion for bromadiolone (509.08) is the protonated dehydrated pseudomolecular ion. In validation it proved impossible to produce reasonable source yield of the unfragmented pseudomolecular ion.

## 11 Decision Criteria

### 11.1 Performance Mix Suitability

Proper calibration and sensitivity of the LC/MS (ESI) are demonstrated each day samples are analyzed. The Rodenticide LC/MS Performance Mix (0.05 µg/mL) is used to verify system suitability. Retention times for each analyte should compare favorably with the last performance mix analysis and each analyte should yield correct exact masses ( $\pm 0.005$  m/z for full MS,  $\pm 0.01$  m/z for MSMS, base peak only) for the ions as shown in Table 1. Commercially available standards of bromadiolone are a mixture of orientational isomers, and it is normal for the chromatographic peak for this compound to be asymmetric and exhibit a “shoulder”.

Table 1: Exact MS and MSMS fragment masses for anticoagulant rodenticides

Compound	Full MS Mass(es)	MSMS masses (base peak in bold)
Coumatetralyl	293.117	131.085, 163.039, 175.039
Warfarin	309.112	147.081, 163.039, 251.071
Coumachlor	343.073, 345.070	163.039, 181.042, 285.032
Difenacoum	445.180	189.054, 257.133, 291.102
Bromadiolone	509.075, 511.073	251.071, 277.086, 321.027
Brodifacoum	523.090, 525.088	189.054, 291.102, 335.043

## 11.2 Analyte Suitability

The following criteria are used as guidelines in determining the acceptability of the data produced in this assay. In general, compound identification should be based on a comparison of the chromatography and mass spectrometry for the analyte peak of interest with data from a contemporaneously analyzed reference standard or extracted Positive Control. In most cases, all of the below should be met in order to identify a target analyte within a biological specimen:

### 11.2.1 Chromatography

The peak of interest should show good chromatographic fidelity, with reasonable peak shape, width, and resolution. In order to be determined acceptable, a chromatographic peak in an unknown sample should compare favorably to a chromatographic peak of the same analyte in a known sample analyzed on the same system in the same or subsequent analytical runs. Additionally, the following two criteria should be met.

#### 11.2.1.1 Retention Time

The retention time of the peak should be within  $\pm 5\%$  of the retention time obtained from injection of a reference standard or extracted Positive Control of the analyte of interest.

#### 11.2.1.2 Signal-to-Noise

To justify the existence of a peak, its baseline signal to peak-to-peak noise ratio should exceed 3. Further, the baseline signal for the peak from the sample of interest should be at least 10 fold greater than that for any observed peak at a similar retention time in a Negative Control or solvent blank sample injected just prior to that sample. Signal to noise will normally be evaluated based upon extracted ion profiles for the ion(s) of interest, with a  $\pm 0.01$  m/z extraction window.

### 11.2.2 Mass Spectrometry

The mass spectrum of the analyte of interest should match that of the appropriate reference standard or an extracted Positive Control within a reasonable degree of scientific certainty. See Table 1 for a list of the ions to be evaluated for each target analyte.

### 12 Calculations

Not applicable.

### 13 Uncertainty of Measurement

Not applicable.

### 14 Limitations

a. Figures of Merit:

Compound	Warfarin	Coumachlor	Coumatetraly 1	Difenacoum	Bromadiolone	Brodifacoum
LOD (note 1)	10 ng/mL	5 ng/mL	2 ng/mL	2 ng/mL	5 ng/mL	2 ng/mL
Recovery (at 10 ng/mL)	9%	34%	23%	69%	58%	59%
Recovery (at 100 ng/mL)	25%	74%	45%	94%	78%	71%
MS Supression (at 10 ng/mL)	75%	90%	76%	73%	79%	63%
MS Supression (at 100 ng/mL)	22%	36%	17%	47%	38%	31%
MSMS Supression (at 10 ng/mL)	37%	56%	46%	52%	17%	60%
MSMS Supression (at 100 ng/mL)	12%	15%	-11%	33%	5%	25%
Stability (note 2)	-33.3%	-5.6%	-15.8%	+24.7%	+6.3%	+7.2%

Note 1: Limits of detection were determined solely from MSMS data.

Note 2: Change in normalized signal for 100 ng/mL positive controls 11 days after analysis vs. day of original analysis. Samples were refrigerated in darkness for the interim.

- b. Interferences: High levels of alprazolam may lead to false negative results for warfarin. Grossly decomposed or putrefied samples may affect limits of detection.

## 15 Safety

Take standard precautions for the handling of chemicals and biological materials. See the *FBI Laboratory Safety Manual* for further guidance.

## 16 References

Felice, L and Murphy, M.J., “The Determination of the Anticoagulant Rodenticide Brodifacoum in Blood Serum by Liquid Chromatography with Fluorescence Detection”, *Journal of Analytical Toxicology* 13: 229-231 (1989).

Grobosch, T, et al., “Acute Bromadiolone Intoxication”, *Journal of Analytical Toxicology* 30: 281-286 (2006).

“Extraction of THC and THC Metabolite from Blood Using Certify II”, Publication from Varian Sample Preparation Products, Harbor City, California.

*Preparation of Chemical Reagents* (Tox 103); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*Analysis of Blood and Urine Specimens for THC and 11 COOH THC* (Tox 405); FBI Laboratory Chemistry Unit – Toxicology Subunit SOP Manual.

*FBI Laboratory Safety Manual*.

Rev. #	Issue Date	History
0	10/27/06	New document.
1	01/19/12	Converted to high resolution MS, and added MSMS analysis, changing: title and sections 2, 3, 5, 10, and 11. Rewrote section 1 to include information about history and pharmacological action of target analytes. Added coumachlor, coumatetraly, difenacoum, and warfarin to targetanalyte list, changing: title and sections 2, 3, 6, 10, 11, and 14. Added additional validation data to comply with current Laboratory Division guidelines, changing: sections 11 and 14. Reduced sample volume used to 1 mL, changing: sections 4, 6, and 9. Updated procedural terminology to match current Chemistry Unit usage, changing: sections 2, 3, 5, 9, and 11. Revised "bench sheet" (appendix 1) for procedural revisions, and added an appendix 2 with instrument parameters checklist.

**Approval**

Redacted - Signatures on File

**Appendix 1: Abbreviated version of the Rodenticide Procedure for bench use.**

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**Appendix 2: Abbreviated version of the Rodenticide instrumental conditions for bench use.**

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